AD_____

Award Number: W81XWH-07-1-0305

TITLE: Innovative Microsystems: Novel Nanostructures to Capture Circulating Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Yitshak Zohar, Ph.D.

CONTRACTING ORGANIZATION: University of Arizona
Tucson, AZ 85721

REPORT DATE: May 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
22-05-2008	Annual	23 APR 2007 - 22 APR 2008

4. TITLE AND SUBTITLE
Innovative Microsystems: Novel Nanostructures to Capture Circulating Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0305

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Yitshak Zohar, Ph.D.

Email: zohar@ame.arizona.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
Tucson, AZ 85721

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to develop a microsystem for sorting metastatic breast cancer cells from a heterogeneous suspension of cells circulating in the blood stream. Conceptually, the technique requires the transformation of a distinguishing biochemical characteristic of the target cells, such as up-regulated cadherin phenotype, into a mechanical or electrical that makes it possible to selectively manipulate the cells on the microscale. The project includes developments of a model system of cells to evaluate cadherin-mediated cell sorting and an integrated bio-functional microfluidic system to capture target cells from heterogeneous suspensions of cells. We have succeeded in the transfection of MDA-MB-231 cells with an N-cadherin expression vector deriving a homogeneous population. An anti-N-cad functionalized surface has been shown to capture N-cad expressing prostate cancer cells (PC3N) with high degree of selectivity. An assay to characterize and a technique to control the amount of immobilized anti-N-cad antibodies on surfaces have been developed to maximize the cell capture efficiency. Microchannels with anti-N-cad functionalized surfaces have been fabricated. Under flow conditions, the capture rate is poor; however, after 15min of incubation time, the capture rate is high. Once captured, the cell/surface adhesion bond is strong enough to sustain high flow-induced shears stress.

15. SUBJECT TERMS
Circulating breast cancer cells, Microfluidics, Nanostructures, Surface derivatization

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	23	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

The goal of this project is to develop a microsystem for continuous sorting of target cells from a heterogeneous suspension of cells. Conceptually, the technique requires the transformation of a distinguishing biochemical characteristic of the target cells, such as up-regulated cadherin phenotype, into a mechanical, electrical or magnetic property that makes it possible to selectively manipulate the cells on the microscale. Such manipulations, then, permit one to continuously flow the entire suspension through a network of nanostructures, sorting out the target cells by taking advantage of their unique physical feature. The project involves the following major steps:

- Determining the parameters of cadherin-mediated adhesion for sorting a single group of cells from a mixed population including the development of a model system of cells to evaluate cadherin-mediated cell sorting on MEMS, and estimating the sensitivity of cadherin-mediated cell capture for an *in vivo* system (SCID mice).
- Fabricating bio-functional microchannels to capture target cells including immobilization of prescribed patterns of cadherin counter-ligands within microchannels etched into silicon, as well as evaluating the effectiveness of these patterns in capturing target cells from flowing suspensions.
- Building an integrated microdevice to select target cells from heterogeneous suspensions of cells.

The first two steps are formulated to lay the foundation for the third step, which involves fabrication of microdevices with integrated networks of microchannels, fluid reservoirs, pumps and the functionalized surface regions.

Body

The results obtained thus far are classified into four topics: (i) development of a model system of cells to evaluate cadherin-mediated cell sorting, (ii) fabrication of microchannels with antibody-functionalized surfaces, (iii) selective binding of breast cancer cells on antibody-coated surfaces, (iv) capture of breast cancer cells in microchannels with antibody-functionalized surfaces, and (v) characterization and manipulation of the amount of antibody on functionalized surfaces.

I - Development of a model system of cells to evaluate cadherin-mediated cell sorting

Our proposal focuses on using N-cadherin to select and sort metastatic breast cancer cells. We utilized the breast cancer cell line MDA-MB-231 as our model system, as it is widely used as a model of metastatic disease. Importantly for our system, MDA-MB-231 cells endogenously express cadherin-11, so we could transfect the cells with a N-cadherin expression vector and compare the capture of transfected versus control cells to test the selectivity of the microdevice. We transfected the NCADGFP vector (pEGFN1, Clontech) into MDA-MB-231 cells and selected stable transfectants with neomycin, resulting in the expression of a human N-cadherin-GFP fusion protein. We utilized a fluorescence activated cell sorter to isolate MDA-MB-231 cells that were GFP positive and over expressing the N-cadherin-GFP fusion protein. After two rounds of sorting, we derived a homogeneous population as evident in Figure 1.

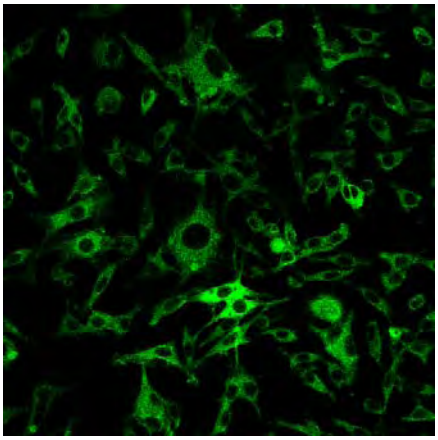
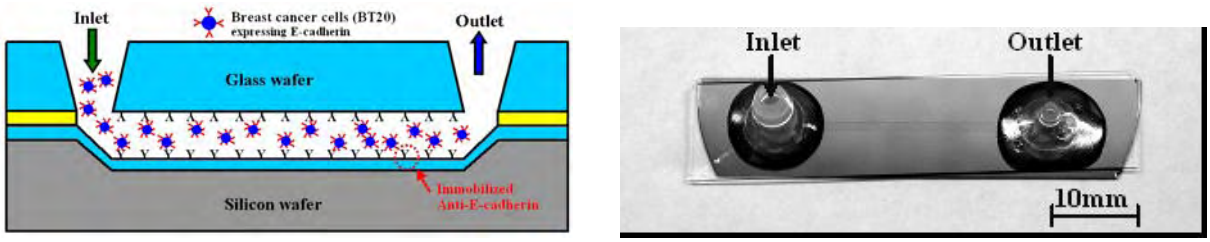


Figure 1. A picture of N-cadherinGFP expressing MDA-MB-231 cells; the green color marks the location of the N-cadherin molecules indicating successful transfection.

Our approach will be to utilize two counter ligands for selecting cell populations. The first is an N-cadherin antibody that binds to the extra cellular domain of N-cadherin. The second is to produce a recombinant N-cadherin counter ligand that consists of the N-cadherin extracellular domain fused to the Fc portion of mouse IgG2b. The full length N-cadherin plasmid described above was used as the template and, by amplifying two PCR fragments, we have cloned the entire extracellular domain (740 amino acids) including the signal peptide, the propepetide and the five extracellular cadherin repeats. These fragment were ligated to the Fc fragment of pBSgamma which we revceived from Dr. R. Mege INSERM. The next step is to subclone the entire fragment into a mammalian expression vector pCEP4 for expression of the fusion protein.

II - Fabrication of microchannels with antibody-functionalized surfaces

A schematic cross-section of an antibody-coated microchannel is illustrated in Figure 2(a), and a photograph of a packaged device with a bio-functionalized microchannel is shown in Figure 2(b). The fabrication of the microchannel starts with a thermal growth of a 0.3μm thick oxide layer on a 4" <100> P-type silicon wafer about 500μm in thickness. The microchannel pattern, is transferred to the oxide etch mask utilizing standard photolithography and etching techniques; the microchannel length is about L=32mm and its width about W=1mm. The microchannel grooves, 100μm deep, are etched in the silicon wafer using 25% TMAH. After stripping the oxide etch mask, a fresh 0.3μm-thick silicon dioxide layer is thermally grown on each processed wafer. In order to maximize the hydroxyl groups on the oxide-coated surface, the substrate is treated with 1:1:6 of 50% hydrogen chloride, 30% hydrogen peroxide and DI water at 80ºC for 15minutes, rinsed in DI water, and dried with nitrogen gas flow.



(a) (b)

Figure 2. (a) A schematic cross section of a microdevice with an antibody-functionalized microchannel and (b) a photograph of a packaged microdevice after completing the fabrication process.

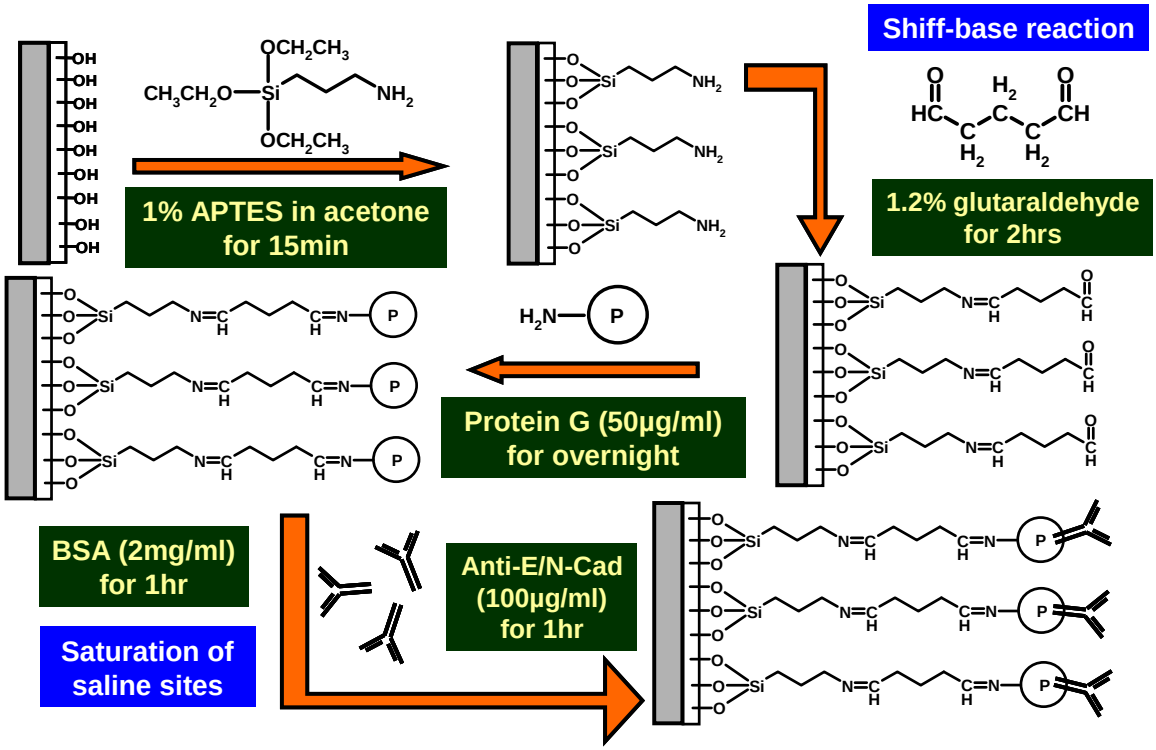
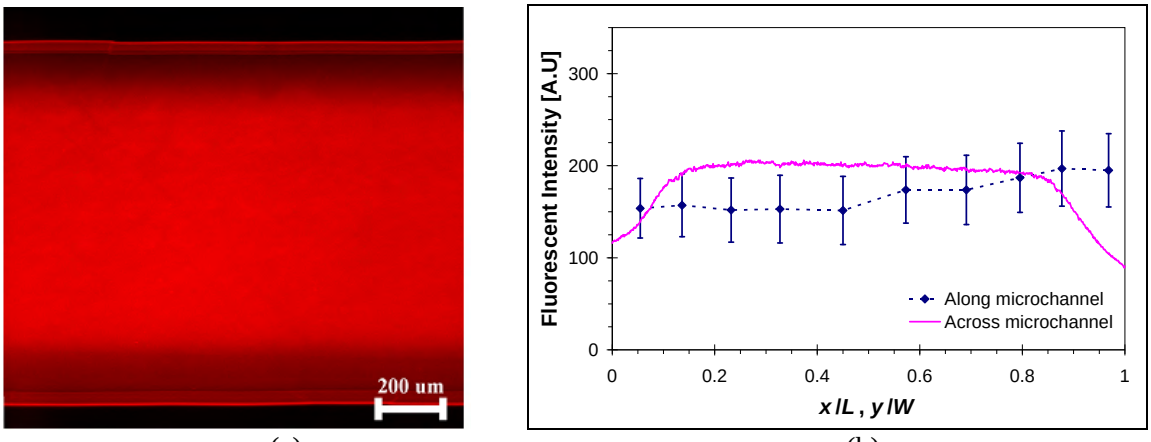


Figure 3. A schematic illustration of the immunoassay for antibody immobilization on an oxide surface.

The immunoassay for functionalizing an oxide surface with antibodies is schematically illustrated in Figure 3. The inner surface of the fabricated microchannel is uniformly functionalized with antibody molecules following a previously reported bio assay [11]. The microchanel is filled with 1% (vol/vol) 3-aminopropyltrioxysilane (APTES)-acetone solution for 15minutes at room temperature. Thus, the hydroxyl groups of the silicon dioxide surface are silanted resulting in self-assembled APTES molecules. The APTES-coated surface is activated with 1.2% (vol/vol) glutaraldehyde in water (Fluka) for 2 hours at room temperature to promote a Schiff-base reaction between the amine and aldehyde groups. After flushing the microchannel with excess of DI water and 1× phosphate buffer saline (PBS) buffer solution, Protein G from *E.coli* (Zymed Lab Inc.), 50 μg/ml in 1×PBS, is incubated on the activated surface for overnight at 4ºC. In order to block excess of silane sites, the channel is filled with bovine serum albumin (BSA) solution (2mg/ml in 1×PBS) for 1 hour at room temperature. Finally, either anti-E-cadherin (anti-E-cad from Zymed Lab Inc) or anti-N-cadherin (anti-N-cad from Sigma) antibodies from mouse, 100 μg/ml in 1×PBS are incubated on the protein G layer at room temperature for 1 hour. The immunoassay is completed after thoroughly washing the microchannel in PBS solution and, thus, obtaining a microchannel inner surface uniformly coated with an antibody layer.

The bio-activity of the antibody layer inside the microchannel, after device packaging, is tested using antibody-antigen interaction. Anti-mouse IgG whole molecules (Jackson ImmunoResearch), tagged with Cy3 fluorescent dyes, at a concentration of 15 μg/ml in 1×PBS are incubated in the microchannel for 1 hour at room temperature in the dark to prevent photo bleaching of the Cy3 molecules. After incubation, the microchannel is washed thoroughly and filled with 1×PBS. The device is then excited under a fluorescent microscope (Nikon eclipse 80i) equipped with Cy3 filter. A red light is emitted from the regions corresponding to the immobilized antibody, as demonstrated in Figure 4(a). The corresponding normalized intensity profiles, plotted in Figure 4(b), are fairly uniform along and across the microchannel (error bars indicate one standard deviation). Thus, the microchannel fabrication process employed in this work leads to the successful immobilization of an anti-E-cad antibody layer still bio-active after device packaging.

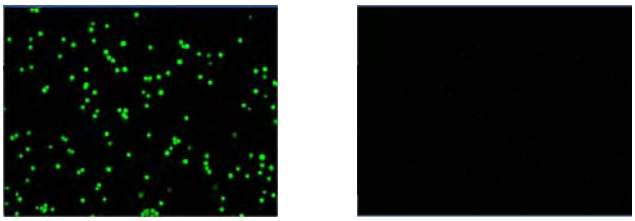


(a) (b)

Figure 4. (a) A fluorescent microscope image of a microchannel segment, about 1mm×1mm in area, following labeled-antigen/antibody interaction to test the bioactivity of the immobilized antibody layer, and (b) the measured intensity profiles along and across the microchannel shown in Figure 4(a).

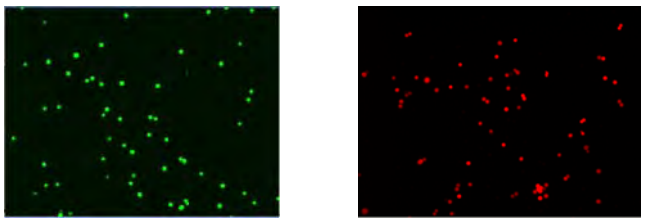
III - Selective binding of breast cancer cells on antibody-coated surfaces

Selective binding of target cells to functionalized surfaces has been tested by incubating a mixture of E-cadherin expressing breast cancer cells, BT20 labeled in red (10^5 cells/ml), and N-cadherin expressing prostate cancer cells, PC3N labeled in green (10^5 cells/ml), on functionalized surfaces. In the first experiment, anti-N-cad antibodies were immobilized on flat silicon wafers. After one hour incubation time of the cell mixture, the samples were washed and images of the attached cells were taken under a fluorescent microscope as sown in Figure 5. The experiment was then repeated on surfaces functionalized with anti-E-cad antibodies, and images of the attached cells are shown in Figure 6.



(a) (b)

Figure 5. Fluorescent microscope images taken after 1 hour incubation of BT20/PC3N mixture on an anti-N-cad surface: (a) using green filter showing many attached N-cad expressing PC3N cells, and (b) using red filter showing practically no attached E-cad expressing BT20 cells.



(a) (b)

Figure 6. Fluorescent microscope images taken after 1 hour incubation of BT20/PC3N mixture on an anti-E-cad surface: (a) using green filter showing attached N-cad expressing PC3N cells, and (b) using red filter showing slightly more attached E-cad expressing BT20 cells.

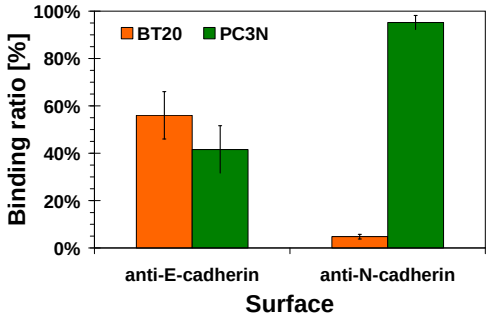


Figure 7. Percentage of captured cells from a BT20/PC3N mixture on flat silicon wafers functionalized with either anti-E-cad or anti-N-cad antibodies after 1 hour incubation time.

The capture selectivity of N-cad expressing cells (PC3N) by the anti-N-cad functionalized surface is very high, as shown in Figure 7; with no hindrance in capture efficiency due to the presence of E-cad expressing cells (BT20). However, the capture selectivity of the anti-E-cad functionalized surface is not so high; a preliminary result that requires further investigation.

IV - Capture of breast cancer cells in microchannels with antibody-functionalized surfaces

The E-cadherin expressing BT20 breast cancer cells were maintained and grown in 1×DMEM/F12 (Invitrogen) with 10% fetal bovine serum (Cellgro) and 1% penicillin-streptomycin (Invitrogen) at 37°C in 5% CO_2 in humid environment. The growth medium wa aspirated and the cells were incubated with 4mM EDTA in 1×calcium free PBS (CMF-PBS) for 15mins for detachment. After centrifugation and solution removal, the cells were re-suspended in 1×S-MEM (Invitrogen) with 0.1% BSA, while the concentration was estimated by cell counting with a haemocytometer. The BT20 cell suspension was loaded into the microchannel using a syringe pump. Once the channel was full, the pump was turned-off allowing cell incubation for a selected time interval. Then, a second syringe pump was used to flush the channel with 1×CMF-PBS, under controlled flow rate, for the removal of un-bounded cells. All experiments were conducted and monitored under a microscope equipped with a CCD camera and a DVD recorder for further data processing as illustrated in Figure 8.

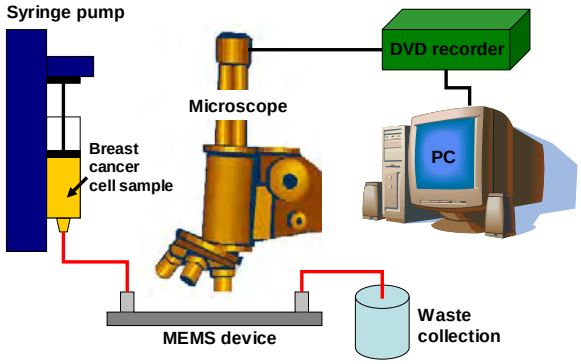


Figure 8. A schematic of the experimental set up including the syringe pump and microfluidic device.

To demonstrate specific cell binding, E-cadherin expressing breast cancer cells, BT20, have been incubated in microchannels coated with either anti-N-cad or anti-E-cad antibodies. After 15min incubation time at room temperature, under no-flow condition, the total number of cells in each microchannel was counted. The microchannels were then washed with 1×CMF-PBS to remove un-bounded cells, leaving inside only BT20 cells firmly attached to the channel surface. The percentage of captured cells in comparison to the initial number of cells present in each channel is plotted in Figure 9; 50% of the cells initially present in the microchannel are captured on the anti-E-cad surface, while less than 10% are captured on the anti-N-cad surface.

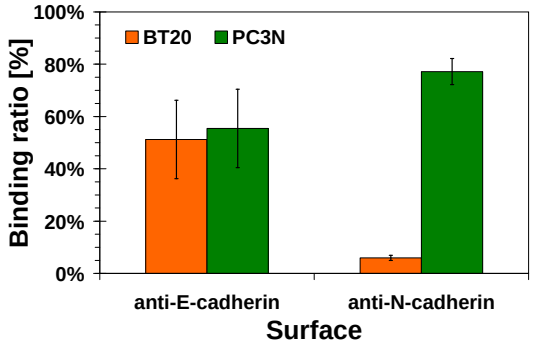
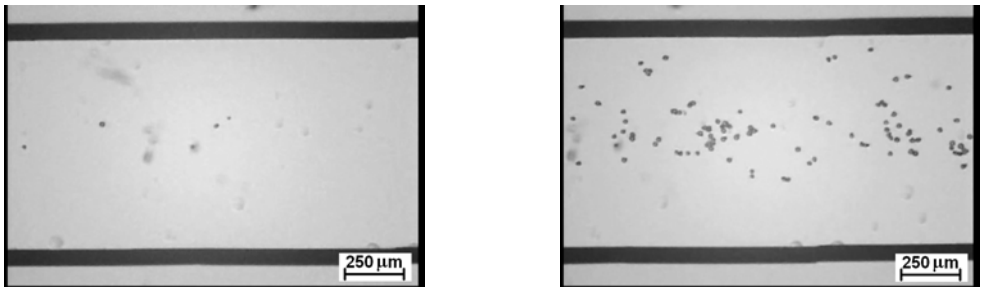


Figure 9. Percentage of captured BT20 and PC3N from homogeneous suspensions in microchannels functionalized with either anti-E-cad or anti-N-cad after 15min incubation time under no-flow condition.

Furthermore, the images in Figure 10 demonstrate that the number of captured BT20 cells by the anti-E-cad coating strongly depends on the incubation time. As shown in Figure 11, increasing the incubation time from 5 to 10 and 15min resulted in a roughly linear increase in the percentage of captured BT20 cells with respect to the initial number of cells loaded into the microchannel.



(a) (b)

Figure 10. Images of captured BT20 breast cancer cells in microchannels coated with anti-E-cadherin after: (a) 5min, and (b) 15min incubation time.

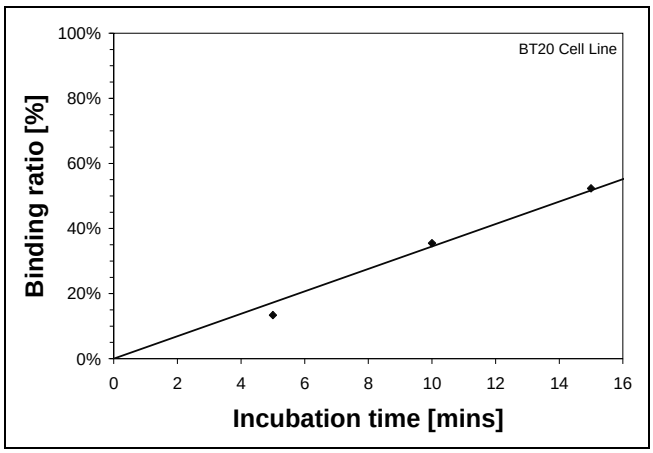
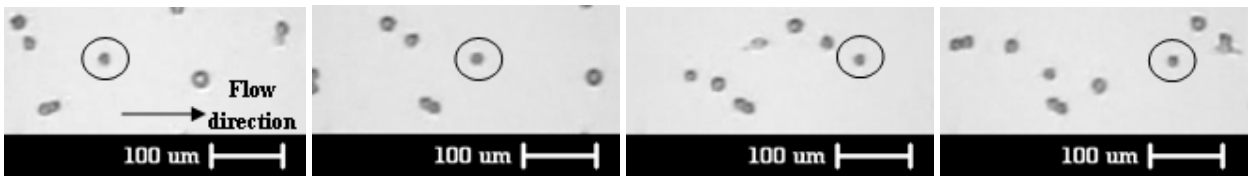


Figure 11. The effect of incubation time on the percentage of cancer cells captured on the anti-E-cad-coated micro-channel surface.

Attempts to capture moving cancer cells at high flow rate were unsuccessful. However, the sequence of images in Figure 12 shows that the location of the marked cell in Figures 12(a-b) is changing, while in Figures 12(c-d) it is fixed. This clearly demonstrates that a moving BT20 cell in contact with an anti-E-cad coated surface under very low flow rate can be captured.



(a) (b) (c) (d)

Figure 12. A time sequence of images showing BT20 cancer cell passing through a microchannel with an anti-E-cad-coated surface: (a) t=0s, (b) 6s, (c) 12s, and (d) 18s.

The time-dependent location of the BT20 cell marked in Figure 12 is plotted in Figure 13 with the slope of the curve being the instantaneous cell velocity. The cell is moving at a constant speed of about 15µm/s with a very short deceleration period/distance before completely stopping.

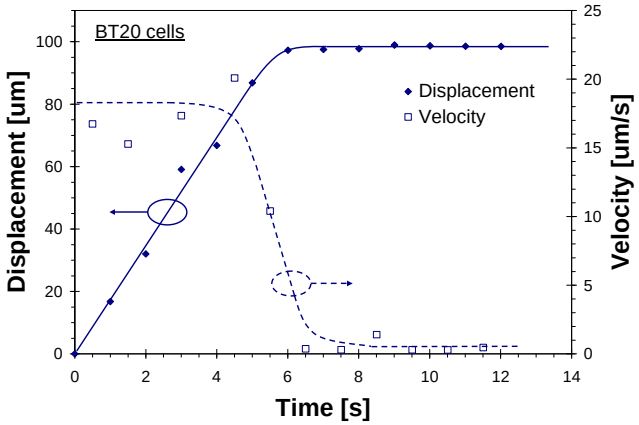
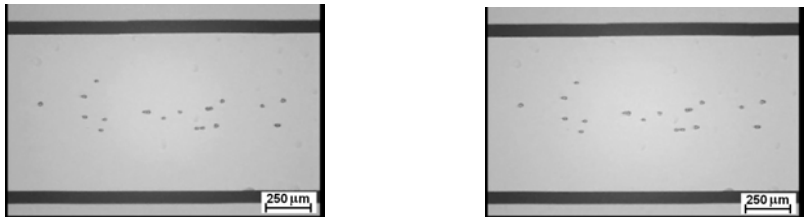


Figure 13. The displacement and velocity of the captured BT20 cancer cell tracked in figure 9.

However, once a strong binding is established, typically after 15min incubation time, flow induced shear stress with average velocity in access of 200mm/s failed to detach the captured cells as evident in the images shown in Figure 14. Zoom in pictures of a captured BT20 cell, shown in Figure 15, indicate that the cell undergoes severe distortion when subjected to high flow rate. Initially spherical about 20µm in diameter, it is elongated to about 40µm in the flow direction due to the high flow rate.



(a) (b)

Figure 14. Images of captured cancer cells on the bottom of a microchannel coated with anti-E-cadherin subjected to flow of (a) 111mm/s, and (b) 222mm/s.

(a) (b)

Figure 15. A captured BT20 cancer: (a) after incubation under no flow, and (b) after capture subject to a flow of 55mm/s.

V. Manipulation and measurement of anti-N-cadherin (ACAM) surface concentration

One important aspect of engineering a microdevice to capture CTCs is the development of methods to derivative surfaces with biologically-active molecules. To engineer the system, we must be able to measure and control the number density of the molecules immobilized on silicon dioxide surfaces. In this sub-section, we focus on the anti-N-cad molecule, also known as ACAM. The surface derivatization and characterization methods developed to this point can be readily extended to the anti-E-cad molecules that we have also used to capture cells (Figures 7, 8, and 10).

To estimate the amount of ACAM on a surface, we have developed an assay that uses a secondary antibody, labeled with horseradish peroxidase (HRP). The antibody attaches to ACAM in a one to one ratio and the conjugated HRP is subsequently used to catalyze the hydrogen peroxide oxidation of a fluorometric substrate (Ultra Amplex Red from Molecular Probes, Inc.) dissolved in an aqueous buffe. The oxidation reaction scheme is illustrated in Figure 16. The fluorescent signal thus generated is used to quantify the amount of ACAM on the derivatized surface.

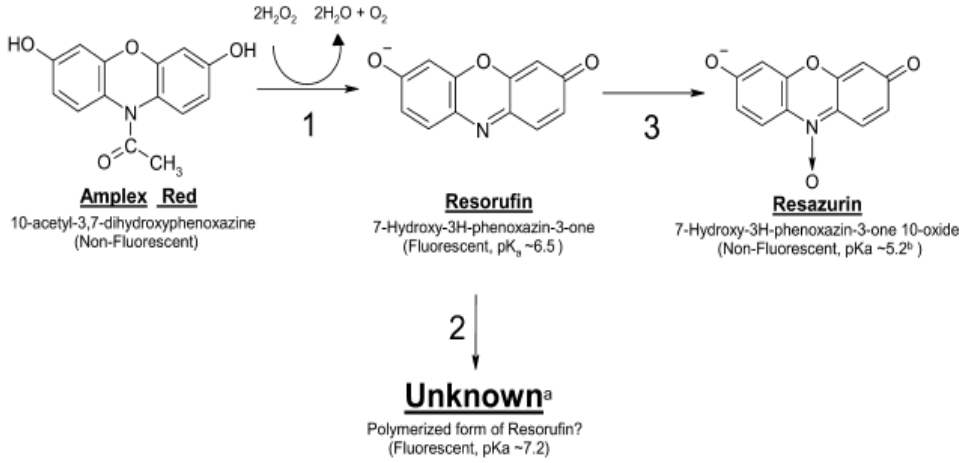


Figure 16. The reaction path for oxidation of Ultra Amplex Red (10-actetly-3,7-dihydroxyphenoxazine) by hydrogen peroxide (H_2O_2). The oxidation process is catalyzed by HRP and yields the fluorescent product Resorufin (7-Hydroxy-3H-phenoxazin-3-one). Resorufin can be subsequently oxidized to form non-fluorescent products [12], but this process happens slowly and does not interfere with properly implemented fluorometric assays.

The assay is performed on 1-cm^2 silicon wafers, derivatized with ACAM by the protocol outlined in Figure 3; we euphemistically refer to these small, square wafers as chiclets. The secondary antibody, viz. rat anti-mouse monoclonal IgG1 istotype labeled with HRP, is then incubated on the chiclet surface for 1 hour. Chiclets are then washed with 1XPBS and individually placed in the wells of a 24-well micro-plate (Fisher Scientific). A working solution comprised of 50μM Ultra Amplex Red, 200 μM hydrogen peroxide in 1XPBS [13-15] is added to the wells and the fluorescent signal is analyzed using the SPECTRAmax Gemini (Molecular Probes). The fluorescence signal, generated by the action of the HRP immobilized on the chiclets, is then read in kinetic mode with excitation and emission max of 444nm and 590nm,

respectively, with an auto cutoff of 590nm. Readings are taken every 60 seconds and the microplate is agitated by the auto shake function 5 seconds before each read. These data are then interpreted with a Michaelis-Menten model of the HRP enzyme kinetics, viz.

$$\frac{dC_p}{dt} = \frac{V_{max} C_s}{K_M + C_s}$$
$$\frac{dC_s}{dt} = -\frac{V_{max} C_s}{K_M + C_s} .$$

Here C_s and C_p respectively denote the concentration of substrate (Ultra Amplex Red) and fluorescent product (Resorufin) in a given micro-plate well as a function of time t; V_{max} and K_M are the standard Michaelis-Menten kinetic parameters. Using a least-squares regression to match the kinetic model to the measured fluorescent signal, we extract values for V_{max} and K_M. As is well known, V_{max} is the parameter related to the amount of enzyme present.

The relationship between V_{max} and the amount of enzyme present is determined empirically by performing the previously described experiment in free solution, absent the chiclets. That is, known amounts of HRP (conjugated to the secondary antibody) are added to solutions of Ultra Amplex Red and hydrogen peroxide and the reaction kinetics are followed. A quantitative relationship between the fluorescent signal, V_{max} and the amount of HRP is thereby established. Figure 17 shows the fluorescent signal generated by 5 ng of antigen (secondary antibody) HRP conjugate dissolved in an individual micro-plate well containing 800μL of Ultra Amplex Red/H_2O_2 solution. An example of an empirically determined calibration curve, which relates V_{max} to the amount of antigen (secondary antibody) HRP conjugate, is shown in Figure 18.

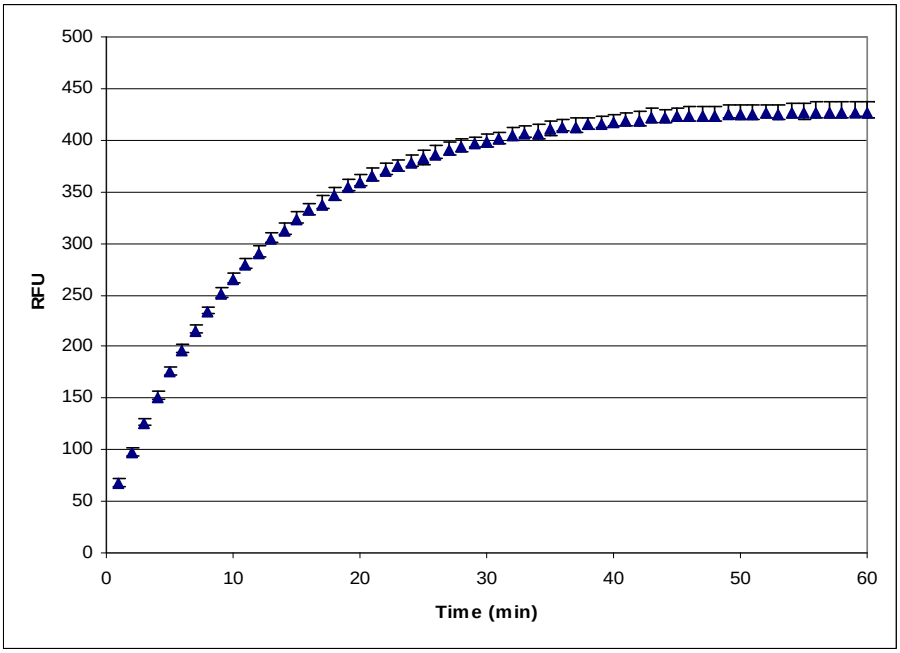


Figure 17. Relative fluorescent units (RFU) generated by 5ng of antigen (secondary antibody) HRP conjugate dissolved in an individual micro-plate well containing 800μL of Ultra Amplex Red/H_2O_2 solution. Averages and error bars are determined from 3 independent experiments.

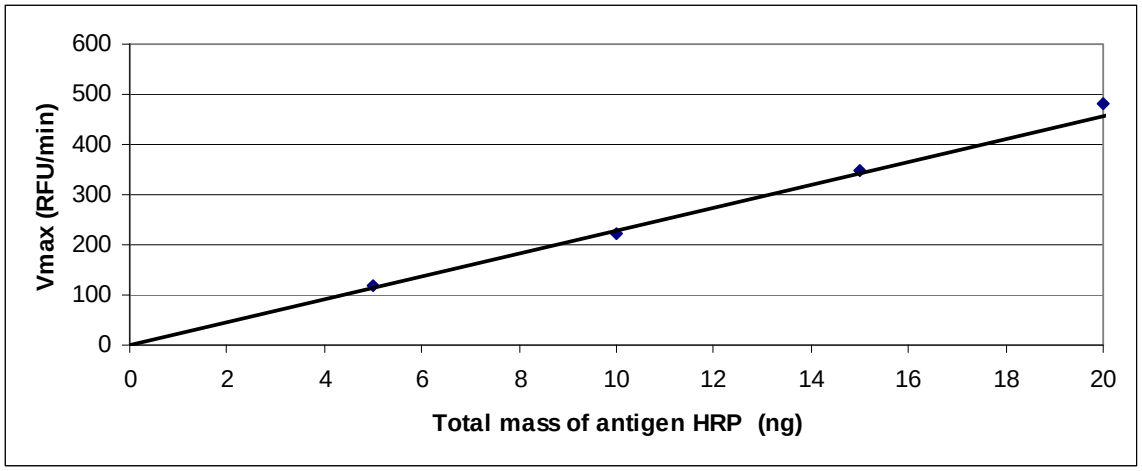


Figure 18. A calibration curve for V_{max}, expressed in relative fluorescent units (RFU) per minute, versus total mass of antigen (secondary antibody) HRP conjugate present in 800μL of buffer in an individual micro-plate well.

In Figure 19 we show a typical fluorescent signal generated by a chiclet, immersed and analyzed in a micro-plate well, as described above. Note the qualitative similarity between the signal generated by HRP immobilized on the chiclet and the signal generated by the HRP in free solution (Figure 17). For these particular results, ACAM was incubated over the chiclet surfaces at an initial concentration of 0.001 mg/ml, which is a slight modification of the protocol summarized in Figure 3.

Signals such as those depicted in Figure 19 are then analyzed to extract values of V_{max}. For example, V_{max} for the signal shown in Figure 19 is 38.5RFU/min. The value for V_{max} is in turn compared to a calibration curve, such as Figure 18, to establish the amount of antigen HRP present on the chiclet. For the chiclet(s) associated with the signal(s) shown in Figure 19, this turns out to be about 1.6 ng/cm^2.

Since the antigen binds to ACAM in a one-to-one arrangement, we then know the amount of ACAM on the chiclet. In Figure 20 we show the number density of ACAM molecules attached to a chiclet surface, after one hour of incubation time, as a function of the initial concentration of ACAM in the incubation solution. For the conditions explored thus far, the results indicate that the amount of ACAM bound to the surface varies linearly with the amount of ACAM initially in solution. Since the ACAM attaches to protein G (Figure 3), the implication of these results is that, for the range of conditions explored, there is a large excess of protein G on the surface.

More importantly, these data show that we can manipulate the amount of ACAM on the derivatized surface by controlling the amount of ACAM in the incubation solution. At an ACAM surface concentration of 2.4×10^9 molecules/cm^2, there is one molecule of ACAM for every 4×10^4 nm^2 of surface area, which corresponds to a patch of surface about 200 nm by 200 nm.

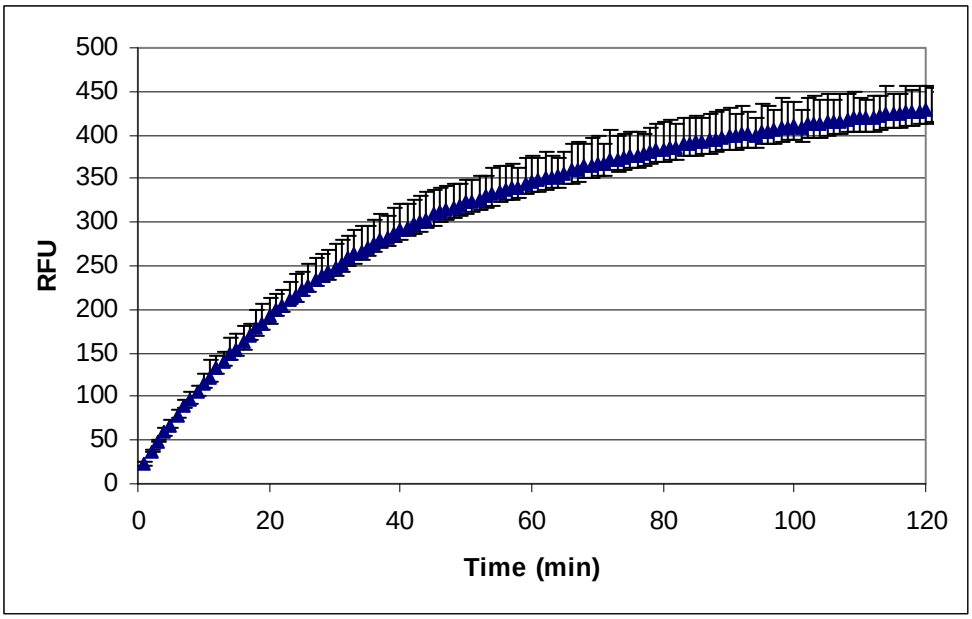


Figure 19. The average fluorescent signal generated by a chiclet as a function of time. Averages are shown for results from independent measurements on six different chiclets. An initial ACAM concentration of 0.001mg/ml was incubated over the chiclet surface. The ratio of antigen (secondary antibody) HRP conjugate concentration to ACAM concentration was on the order of 2 to 5. Here V_{max} is 38.5RFU/min.

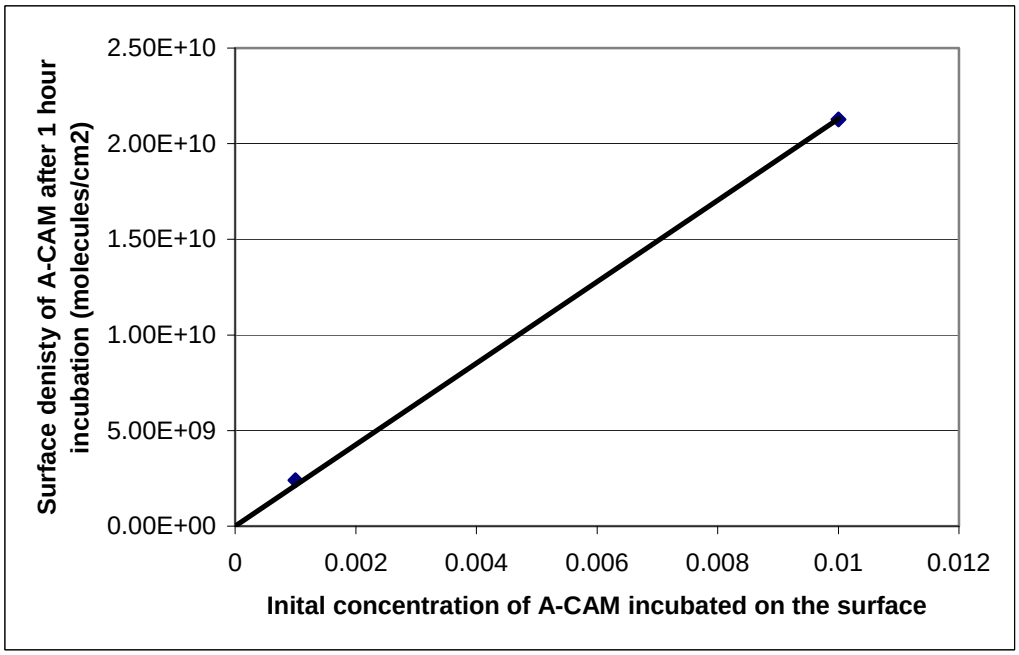


Figure 20. The density of ACAM (molecules/cm^2) attached to a chiclet surface after one-hour incubation time versus the initial ACAM concentration in solution. Measurements were taken at initial ACAM concentrations of 0.001 mg/ml and 0.01 mg/ml; a trend line in included to indicate the remarkably linear relationship between the resultant surface number density and the initial concentration of ACAM in solution.

In summary we have made the following progress with respect to surface modification and characterization during the first year of work. We have developed an assay to quantify the amount of active ligand (in this case ACAM) that we attach to silicon dioxide surfaces. And we have shown that we can control the amount of surface bound ACAM by controlling the initial concentration of ACAM in the solution from which we do the incubation. Another key process parameter that we can use to control the concentration of bound ACAM is the incubation time and these studies are currently underway.

Key Research Accomplishments

- Transfection of MDA-231 to over express an N-cadherin-GFP fusion protein
- Fabrication of microchannels with active antibody-functionalized surfaces
- Assembly of the experimental set-up for driving cells through the microchannels
- Demonstration of the highly selective binding of N-cad expressing cancer cells to a flat surface functionalized with anti-N-cad antibodies
- Capture of cancer cells in functionalized microchannels:
    - Poor capture yield under flow conditions even at very low speed
    - High capture yield after 15min incubation time under no-flow conditions
- Strong attachment of the cancer cells to the functionalized microchannels; high flow-induced shear stress not adequate for subsequent cell detachment
- Developed assay to characterize the amount of anti-N-cad antibodies on derivatized surfaces
- Demonstrated that we can control amount of anti-N-cad antibodies on derivatized surfaces by manipulating the amount of anti-N-cad antibodies in incubation solution

Reportable Outcomes

- L.S.L. Cheung, X.J. Zheng, A. Stopa, J.A. Schroeder, R.L. Heimark, J.C. Baygents, R. Guzman, Y. Zohar, Capture of breast cancer cells in a microfluidic system. *First Sensor, Signal and Information Processing (SenSIP) Workshop*, May 11-14, 2008, Sedona, Arizona.
- L.S.L. Cheung, X.J. Zheng, A. Stopa, J.A. Schroeder, R.L. Heimark, J.C. Baygents, R. Guzman, Y. Zohar, Capture of circulating cancer cells using microfluidic systems. *Fifth Era of Hope Meeting*, Jun. 25-28, 2008, Baltimore, Maryland.

Conclusions

MDA-MB-231 cells have been successfully transfected with a N-cadherin expression vector. Since MDA-MB-231 cells endogenously express cadherin-11, this will allow us to compare the capture of transfected versus control cells to test the selectivity of the microfluidic system. An anti-N-cad functionalized surface has been found to be highly specific in binding N-cad expressing cells, even in the presence of E-cad expressing cell. Therefore, functionalized surfaces can be used as a cell sorting tool. Microfluidic systems with anti-N-cad functionalized surfaces have been fabricated to be used as a sorting device of metastatic prostate cancer cells from heterogeneous suspensions of cells. Thus far, it has been difficult to capture target cells that are in motion; even at low speed; however, 15min of incubation time is sufficient for allowing the target cells to strongly bind to the functionalized surface. Therefore, the microsystem can still be operated in a continuous mode but with an unsteady flow rate. An assay has been developed to characterize the amount of immobilized anti-N-cad antibodies on derivatized surfaces; furthermore, the amount of anti-N-cad antibodies on derivatized surfaces can be controlled by manipulating the amount of anti-N-cad antibodies in the incubation solution. This is an important step in the effort of maximize the capture efficiency of target cells by the functionalized surfaces.

References

[1] M. Cristofanilli, G.T. Budd, M.J. Ellis, A. Stopeck, J. Matera, M.C. Miller, J.M. Reuben, G.V. Doyle, W.J. Allard,L.W.M.M. Terstappen, and D.F. Hayes, "Circulating tumor cells, disease progression, and survival in metastatic breast cancer," *N. Engl. J. Med.*, vol. 351, pp. 781-791, 2004.

[2] H.J. Kahn, A. Presta, L.Y. Yang, J. Blondal, M. Trudeau, L. Lickley, C. Holloway, D.R. McCready, D. Maclean, and A. Marks, "Enumeration of circulating tumor cells in blood of breast cancer patients after filtration enrichment: correlation with disease stage," *Breast Cancer Res. Treast.*, vol. 86, pp. 237-247, 2004.

[3] J.B. Smerage, and D.F. Hayes, "The measurement and therapeutic implications of circulating tumour cells in breast cancer," *Br. J. Cancer*, vol. 94, pp. 8-12, 2006.

[4] V. Zieglschmid, C. Hollmann, and O. Bocher, "Detection of disseminated tumor cells in peripheral blood," *Crit. Rev. Clin. Lab. Sci.*, vol. 42, pp. 155-196, 2005.

[5] W.C. Chang, L.P. Lee, and D. Liepmann, "Biomimetic technique for adhesion-based collection and separation of cells in a microfluidic channel," *Lab Chip*, vol. 5, pp. 64-73, 2005.

[6] M. Toner, and D. Irimia, "Blood-on-a-chip," *Annu. Rev. Biomed. Eng.*, vol. 7, pp. 77-103, 2005.

[7] Z. Du, K.H. Cheng, M.W. Vaughn, N.L. Collie, and L.S. Gollahon, "Recognition and capture of breast cancer cells using an antibody-based platform in a microelectromechanical systems device," *Biomed. Microdevices*, vol. 9, pp. 35-42, 2007.

[8] T. Park, T. Jensen, D. Park, J. Guy, P. Datta, S.A. Soper, and M.C. Murphy, "Capture of very rare circulating tumor cells for human breast cancer diagnosis," *Proc. IMECE2007*, IMECE2007-42425, 2007.

[9] S. Nagrath, L.V. Sequist, S. Maheswaran, D.W. Bell, D. Irimia, L. Ulkus, M.R. Smith, E.L. Kwak, S. Digumarthy, A. Muzikansky, P. Ryan, U.J. Balis, R.G. Tompkins, D.A. Haber, and M. Toner, "Isolation of rare circulating tumour cells in cancer patients by microchip technology," *Nature*, vol. 450, pp. 1235-1239, 2007.

[10] R. Iwama, L.M. Lee, E.S. Cho, and Y. Zohar, "Fabrication of microchannels with patterned bio-active layers," *Proc. MEMS2007*, pp. 333-336, 2007.

[11] L.M Lee, R.L. Heimark, R. Guzman, J.C. Baygents, and Y. Zohar , "Low melting point agarose as a protection layer in photolithographic patterning of aligned binary proteins," *Lab Chip*, vol. 6, pp. 1080-1085, 2006.

[12] V. Towne, M. Will, B. Oswald, Q. Zhao, "Complexities in horseradish peroxidase-catalyzed oxidation of dihydroxyphanoxazine derivatives: appropriate ranges fro pH values and hydrogen peroxide concentrations in quantitative analysis," *Analytical Biochemistry*, vol. 334 pp. 290-296, 2004.

[13] Molecular Probes; Invitrogen dectection company, Amplex Red ELISA Kit #2, with Protein G, Horseradish Peroxidase Conjugate (A22171), MP 22171, 2004.

[14] M. Zhou, Z. Diwu, N. Panchuk-Voloshina, R.P. Haugland, "A Stable Nonfluorescent Derivative of Resourufin for te Flourometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases", *Analytical Biochemistry* vol. 253 pp162-168, 1997.

[15] J.G. Mohanty, J.S. Jaffe, E.S. Schulman, D.G. Raible, A highly sensitive fluorescent micro-assay of H2O2 release from activated human leukocytes using a dihydroxyphanoxazine derivative", *J. Immunol Methods*, vol. 202, pp133-141 ,1997.

Appendices

I - Presented at the *First Sensor, Signal and Information Processing (SenSIP) Workshop*, May 11-14, 2008, Sedona, Arizona

CAPTURE OF BREAST CANCER CELLS IN A MICROFLUIDIC SYSTEM

L.S.L. Cheung[1], X.J. Zheng[1], A. Stopa[2], J. Schroeder[3], R.L. Heimark[4], J.C. Baygents[2], R. Guzman[2], and Y. Zohar[1]

[1]Dept. Aerospace and Mechanical Engineering, [2]Dept. Chemical and Environmental Engineering, [3]Dept. Molecular and Cellular Biology, [4]Dept. Surgery, University of Arizona, Tucson, AZ 85721

ABSTRACT

Circulating tumor cells represent an alternative to invasive biopsies for cancer detection and characterization. Current techniques for isolating these cells are limited to complex analytic approaches with poor results. Here, selective binding of breast cancer cells to a biologically derivatized surface, utilizing a microfluidic system, has experimentally been studied under both static (no-flow) and dynamic (flow) conditions. Silicon-to-glass bonding is used to fabricate a microchannel device followed by immobilization of anti-E-Cadherin molecules on the channel surface. This bio-active coating is indeed highly specific in capturing BT20 breast cancer cells. Furthermore, the number of capture cells increases almost linearly with incubation time within the first 15min. The effect of flow velocity on capturing cancer cells has also been investigated.

Index Terms – Circulating tumor cells, adhesion-based separation, microchannel antibody coating

1. INTRODUCTION

Circulating tumor cells have been identified in peripheral blood from cancer patients and are probably the origin of intractable metastatic disease [1]. These tumor cells are very rare, comprising as few as one cell per 10^9 cells in the blood of patients with metastatic cancer; hence, their isolation presents a tremendous technical challenge [2]. Nonetheless, these cells represent a potential alternative to invasive biopsies as a source of tumor tissue for the detection, characterization and monitoring of non-haematologic cancers [3]. The ability to identify, isolate and molecularly characterize circulating tumor cells subpopulations could further the discovery of cancer stem cell biomarkers and enhance the understanding of the biology of metastasis. Current strategies for isolating cancer cells circulating in the blood stream are still limited to complex analytic approaches that generate very low yield and purity [4].

Fluidic microsystems provide unique opportunities for cell sorting and rare-cell detection; they have been used for microfluidic flow cytometry, continuous size-based separation, and adhesion-based separation [5]. Despite their success in manipulating minute amounts of simple liquids in microscale channels, they have thus far shown limited capability to deal with the cellular and fluid complexity of large volumes of whole blood samples [6]. Recently, the capture of tumor cells has been demonstrated using an antibody-based platform in a microdevice [7]. Micro-channels have been functionalized with anti-epithelial-cell-adhesion molecule (anti-EpCAM) antibodies to generate capture surfaces for collection of target cells [8]. In a landmark study, using the same EpCAM antibody coating, a microchannel with microposts has been developed for selective separation of viable circulating tumor cells from peripheral whole blood samples [9]. The fabricated microdevices identified tumor cells in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast and colon cancer with approximately 50% purity. Furthermore, the chip was utilized in monitoring changes in tumor cells number due to anti-cancer therapy; thus, providing a new and effective tool for accurate identification and measurement of tumor cells in cancer patients. Following a similar approach, we report selective binding of breast cancer cells in a microchannel coated with anti-E-Cadherin antibodies.

2. EXPERIMENTAL ARRANGEMENTS

A schematic cross-section of an antibody-coated microchannel is illustrated in Figure 1, and the device fabrication process has been detailed elsewhere [10]. The fabrication of the microchannel starts with a thermal growth of a 0.3μm thick oxide layer on a 4" <100> P-type silicon wafer about 500μm in thickness. The microchannel pattern, is transferred to the oxide etch mask utilizing standard photolithography and etching techniques; the microchannel length is about L=32mm and its width about W=1mm. The microchannel grooves, 100μm deep, are etched in the silicon wafer using 25% TMAH. After stripping the oxide etch mask, a fresh 0.3μm-thick silicon dioxide layer is thermally grown on each processed wafer. In order to maximize the hydroxyl groups on the oxide-coated surface, the substrate is treated with 1:1:6 of 50% hydrogen chloride, 30% hydrogen peroxide and DI water at 80°C for 15minutes, rinsed in DI water, and dried with nitrogen gas flow.

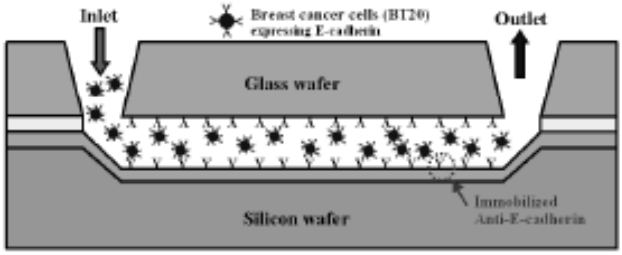


Figure 1. A schematic Cross section of a microdevice with an antibody-functionalized microchannel surface.

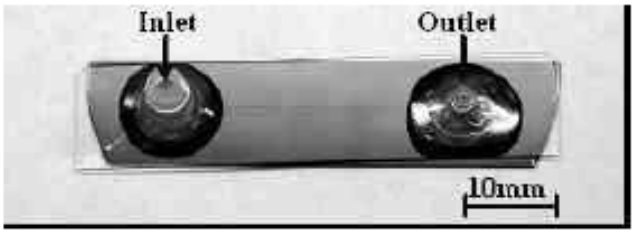


Figure 2. A photograph of a packaged microdevice with microchannel inlet/outlet adaptors after completion of the microchannel fabrication process.

The inner surface of the fabricated microchannel is uniformly functionalized with antibody molecules following a previously reported bio assay [11]. The microchanel is filled with 1% (vol/vol) 3-aminopropyltrioxysilane (APTES)-acetone solution for 15minutes at room temperature. Thus, the hydroxyl groups of the silicon dioxide surface are silanted resulting in self-assembled APTES molecules. The APTES-coated surface is activated with 1.2% (vol/vol) glutaraldehyde in water (Fluka) for 2 hours at room temperature to promote a Schiff-base reaction between the amine and aldehyde groups. After flushing the microchannel with excess of DI water and 1× phosphate buffer saline (PBS) buffer solution, Protein G from *E.coli* (Zymed Lab Inc.), 50 μg/ml in 1×PBS, is incubated on the activated surface for overnight at 4°C. In order to block excess of silane sites, the channel is filled with bovine serum albumin (BSA) solution (2mg/ml in 1×PBS) for 1 hour at room temperature. Finally, either anti-E-cadherin (anti-E-Cad from Zymed Lab Inc) or anti-N-cadherin (anti-N-Cad from Sigma) antibodies from mouse, 100 μg/ml in 1×PBS are incubated on the protein G layer at room temperature for 1 hour. The immunoassay is completed after thoroughly washing the microchannel in PBS solution and, thus, obtaining a microchannel inner surface uniformly coated with an antibody layer. A photograph of a packaged device, with a functionalized microchannel, is shown in Figure 2.

The bio-activity of the antibody layer inside the microchannel, after device packaging, is tested using antibody-antigen interaction. Anti-mouse IgG whole molecules (Jackson ImmunoResearch), tagged with Cy3 fluorescent dyes, at a concentration of 15 μg/ml in 1×PBS are incubated in the microchannel for 1 hour at room temperature in the dark to prevent photo bleaching of the Cy3 molecules. After incubation, the microchannel is washed thoroughly and filled with 1×PBS. The device is then excited under a fluorescent microscope (Nikon eclipse 80i) equipped with Cy3 filter. A red light is emitted from the regions corresponding to the immobilized antibody, as demonstrated in Figure 3. Furthermore, the corresponding normalized intensity profiles, plotted in Figure 4, are fairly uniform along and across the microchannel (error bars indicate one standard deviation). Thus, the microchannel fabrication process employed in this work leads to the successful immobilization of an anti-E-Cad antibody layer still bio-active after device packaging.

The E-cadherin expressing BT20 breast cancer cell line is maintained and grown in 1×DMEM/F12 (Invitrogen) with 10% fetal bovine serum (Cellgro) and 1% penicillin-streptomycin (Invitrogen) at 37°C in 5% CO_2 in humid environment. The growth medium is aspirated and the cells are incubated with 4mM EDTA in 1×calcium free PBS (CMF-PBS) for 15mins for detachment. After centrifugation and solution removal, the cells are re-suspended in 1×S-MEM (Invitrogen) with 0.1% BSA, while the concentration is determining by cell counting with a haemocytometer.

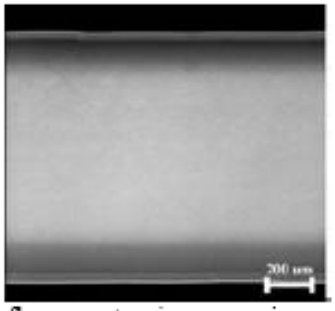


Figure 3. A fluorescent microscope image of a micro-channel segment, about 1mm^2 in area, following labeled-antigen/antibody interaction to test the bioactivity of the immobilized antibody layer.

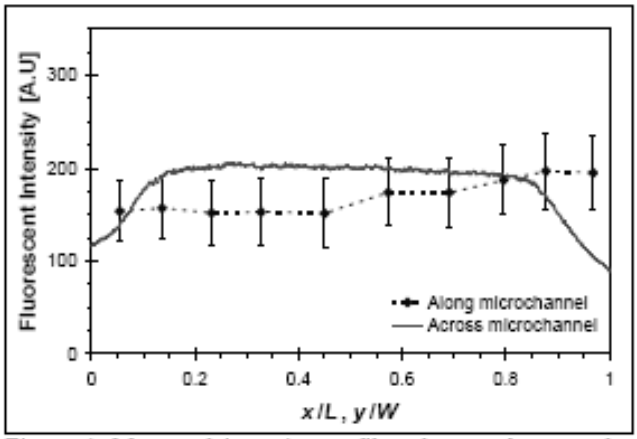


Figure 4. Measured intensity profiles along and across the microchannel shown in Figure 3.

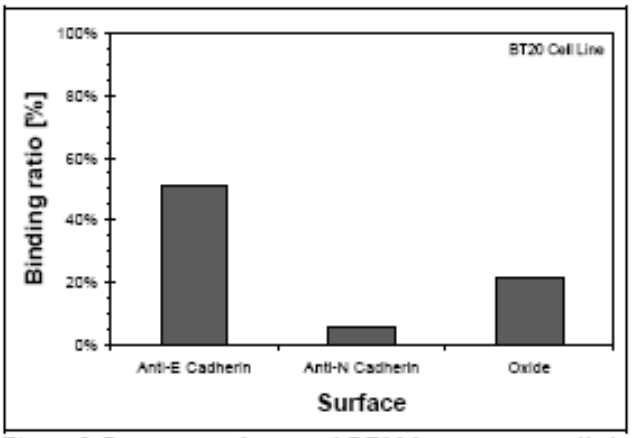


Figure 5. Percentage of captured BT20 breast cancer cells in three microchannels with different surface coating: silicon dioxide, anti-N-Cad and anti-E-Cad antibodies after 15min incubation time under no-flow condition.

The BT20 cell suspension is loaded into the microchannel using a syringe pump. Once the channel is filled, the pump is turned-off allowing cell incubation for a selected time interval. Then, a second syringe pump is used to flush the channel with 1×CMF-PBS, under controlled flow rate, for the removal of un-bounded cells and testing the cell-adhesion strength. All experiments are conducted and monitored under a microscope equipped with a CCD camera and a DVD recorder for further data processing.

3. RESULTS AND DISCUSSION

3.1. Cell capture under no-flow condition

To demonstrate specific cell binding, E-Cadherin expressing breast cancer cells, BT20, have been incubated in three microchannels coated with either silicon dioxide, anti-N-Cadherin or anti-E-Cadherin antibodies. After 15min incubation time at room temperature, under no-flow condition, the total number of cells in each microchannel is counted. The microchannels are then washed with 1×CMF-PBS to remove un-bounded cells, leaving inside only BT20 cells firmly attached to the channel surface. The percentage of captured cells in comparison to the initial number of cells present in each channel is plotted in Figure 5 for the three tested microchannels; 50% of the cells initially present in the microchannel are captured on the anti-E-Cad surface, while less than 10% are captured on the anti-N-Cad surface, and about 20% bind non-specifically on the oxide surface.

Furthermore, the images in Figure 6 demonstrate that the number of captured BT20 cells by the anti-E-Cad coating strongly depends on the incubation time. As shown in Figure 7, increasing the incubation time from 5 to 10 and 15min resulted in a roughly linear increase in the percentage of captured BT20 cells with respect to the initial number of cells loaded into the microchannel.

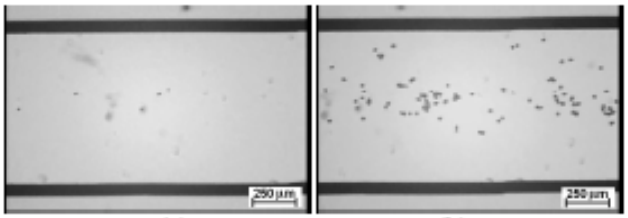


Figure 6. Images of captured BT20 breast cancer cells in microchannels coated with anti-E-Cadherin after: (a) 5min, and (b) 15min incubation time.

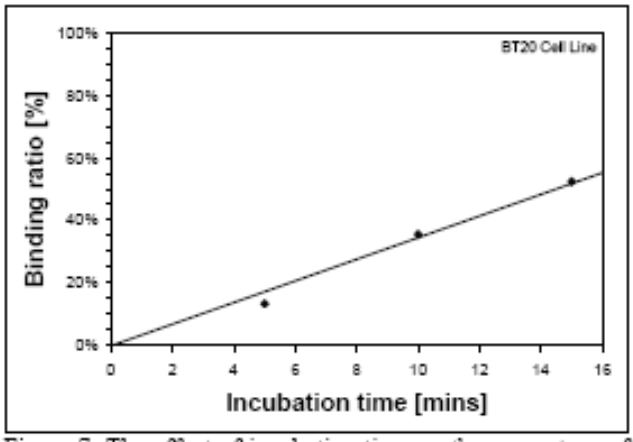


Figure 7. The effect of incubation time on the percentage of cancer cells captured on the anti-E-Cad-coated micro-channel surface.

3.1. Cell capture under flow condition

Attempts to capture moving cancer cells at high flow rate were unsuccessful. However, the sequence of images in Figure 8 shows that the location of the marked cell in Figures 8(a-b) is changing, while in Figures 8(c-d) it is fixed. This clearly demonstrates that a moving BT20 cell in contact with an Anti-E-cad coated surface can be captured.

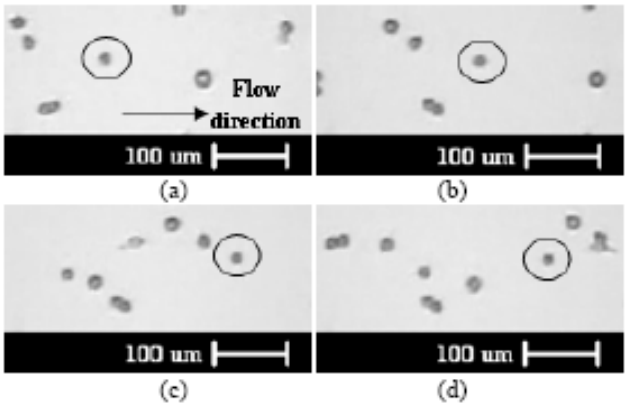


Figure 8. A time sequence of images showing BT20 cancer cell passing through a microchannel with an anti-E-Cad-coated surface: (a) t=0s, (b) t=6s, (c) t=12s and (d) t=18s.

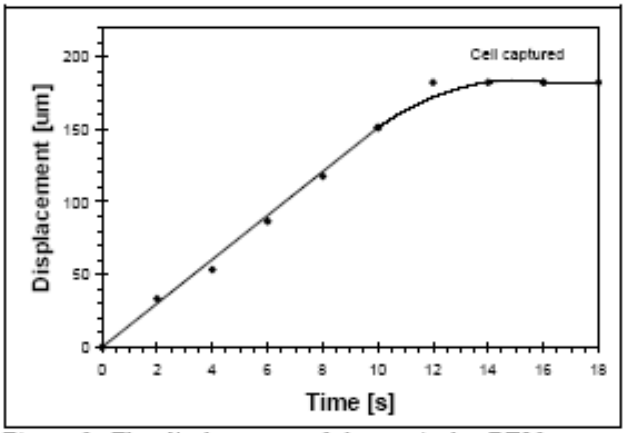


Figure 9. The displacement of the particular BT20 cancer cell tracked in figure 9 indicating capture.

The time-dependent location of the BT20 cell, marked in Figure 8, is plotted in Figure 9 with the slope of the curve being the instantaneous cell velocity. The cell is moving at a constant speed of about 15μm/s with a very short deceleration period/distance before completely stopping.

However, once a strong binding is established, typically after 15min incubation time, flow induced shear stress with average velocity in access of 200mm/s failed to dislodge the captured cells as evident in the images shown in Figure 10. Zoom in pictures of a captured BT20 cell, shown in Figure 11, indicate that the cell undergoes severe distortion when subjected to high flow rate. Initially spherical about 20μm in diameter, it is elongated to about 40μm in the flow direction due to the high flow rate.

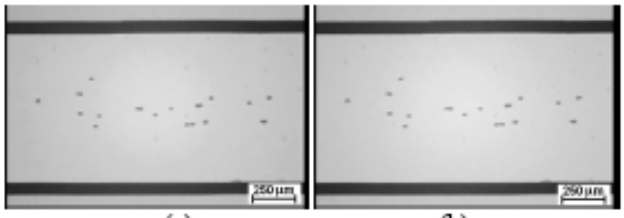


Figure 10. Images of captured cancer cells on the bottom of a microchannel coated with anti-E-Cadherin subjected to flow of (a) 111mm/s, and (b) 222mm/s.

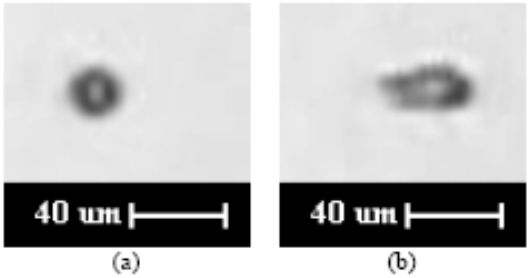


Figure 11. A captured BT20 cancer: (a) after incubation, no flow; and (b) after capture, subject to a flow of 55mm/s.

4. CONCLUSIONS

Microchannels with anti-E-Cad antibody coated surface have been successfully utilized to specifically capture E-cadherin expressing BT20 breast cancer cells. Under no flow condition, the number of captured BT20 cells increases linearly with incubation time. Cells moving at a low speed in contact with the coated surface can be captured, while captured cells can withstand very high flow rates.

ACKNOWLEDGEMENTS

This work is supported by a BCRP grant, BC061859, administered by the US Army Medical Research.

5. REFERENCES

[1] M.Cristofanilli *et al.*, "Circulating tumor cells, disease progression, and survival in metastatic breast cancer," *N. Engl. J. Med.*, vol. 351, pp. 781-791, 2004.

[2] H.J. Kahn *et al.*, "Enumeration of circulating tumor cells in blood of breast cancer patients after filtration enrichment: correlation with disease stage," *Breast Cancer Res. Treast.*, vol. 86, pp. 237-247, 2004.

[3] J.B. Smerage, and D.F. Hayes, "The measurement and therapeutic implications of circulating tumour cells in breast cancer," *Br. J. Cancer*, vol. 94, pp. 8-12, 2006.

[4] V. Zieglschmid, C. Hollmann, and O. Bocher, "Detection of disseminated tumor cells in peripheral blood," *Crit. Rev. Clin. Lab. Sci.*, vol. 42, pp. 155-196, 2005.

[5] W.C. Chang, L.P. Lee, and D. Liepmann, "Biomimetic technique for adhesion-based collection and separation of cells in a microfluidic channel," *Lab Chip*, vol. 5, pp. 64-73, 2005.

[6] M. Toner and D. Irimia, "Blood-on-a-chip," *Annu. Rev. Biomed. Eng.*, vol. 7, pp. 77-103, 2005.

[7] Z. Du *et al*, "Recognition and capture of breast cancer cells using an antibody-based platform in a microelectromechanical systems device," *Biomed. Microdevices*, vol. 9, pp. 35-42, 2007.

[8] T. Park *et al*, "Capture of very rare circulating tumor cells for human breast cancer diagnosis," *Proc. IMECE2007*, IMECE2007-42425, 2007.

[9] S. Nagrath *et al.*, "Isolation of rare circulating tumour cells in cancer patients by microchip technology," *Nature*, vol. 450, pp. 1235-1239, 2007.

[10] R. Iwama *et al*, "Fabrication of microchannels with patterned bio-active layers," *Proc. MEMS2007*, pp. 333-336, 2007.

[11] L.M Lee *et al*, "Low melting point agarose as a protection layer in photolithographic patterning of aligned binary proteins," *Lab Chip*, vol. 6, pp. 1080-1085, 2006.

II - To be presented at the *Fifth Era of Hope Meeting*, Jun. 25-28, 2008, Baltimore, Maryland

CAPTURE OF CIRCULATING CANCER CELLS USING MICROFLUIDIC SYSTEMS

L.S.L. Cheung[1], X.J. Zheng[1], A. Stopa[2], J.A. Schroeder[3], R.L. Heimark[4], J.C. Baygents[2], R. Guzman[2], Y. Zohar[1]
[1]*Dept. Aerospace and Mechanical Engineering,* [2]*Dept. Chemical and Environmental Engineering,* [3]*Dept. Molecular and Cellular Biology,* [4]*Dept. Surgery, University of Arizona, Tucson, AZ 85721, USA*

The use of a microfluidic system to capture metastatic cancer cells from suspension has been studied. Here capture occurs on microchannel surfaces that contain an immobilized cadherin ligand, which specifically binds the target cells, and capture has been studied under both no-flow and flow conditions. Breast carcinoma cells that metastasize to the circulation, in contrast with normal epithelial cells, typically down-regulate E-cadherin and can up-regulate N-cadherin. Hence, the cadherin system provides a natural tool to select metastatic tumor cells circulating in the blood.

The microchannel fabrication process starts with patterning a 3000Å-thick thermal oxide layer on a 2" silicon wafer. A TMAH solution is used to etch channels about 100μm deep and the oxide mask is stripped away. The substrate is then re-oxidized forming a 1μm-thick silicon dioxide layer. Separately, a glass cover slip, with inlet/outlet holes, is made to place over the etched silicon base and form the upper surfaces of the channels. The cover-slip is incubated with PEG solution to prevent non-specific binding on the upper channel surface. UV glue, applied with a fabric brush, is used to bond the cover-slip and the etched-silicon base in a contact mask aligner. Bottom surfaces within the channels are then derivatized as follows. The hydroxyl groups on the oxide surface are silanated in 1% (vol./vol.) 3-aminopropyltriethoxysilane (APTES)-acetone solution, followed by activation in 2% (vol./vol.) glutaraldehyde in phosphate buffer saline. Protein G is next incubated on the activated surface. Finally, anti-N-cadherin IgG or E-cadherin IgG is immobilized on the protein G layer through binding of the Fc region.

To demonstrate cell capture, N-cadherin expressing test cells have been incubated in microchannels coated with either anti N-cadherin or normal IgG antibodies at room temperature for 15min in calcium-free media with 0.1% BSA. After standard washing, 80% of the cells initially present in the channel are captured on the anti-N-cadherin surface under no-flow conditions (Fig. 1). Only 10% are captured on the mseIgG surface. Increasing the incubation time from 5 to 15min increased the percentage of captured cells from 10 to 80%. Once strong binding is established, flow induced shear stress with average velocity of up to 3mm/s failed to dislodge the captured cells. In dynamic conditions, i.e. cell capture from flowing suspensions, cells with a translation velocity as low as 0.1mm/s have been observed to roll on the derivatized surface, though no cells have been captured at even these modest translation rates.

Figure 1: Cancer cells captured in a microchannel, 100μm deep and 1mm wide.